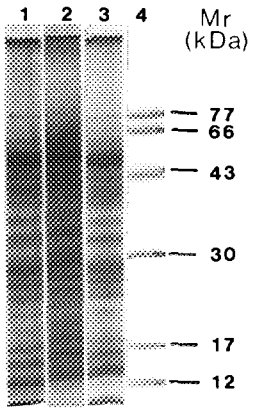

FIG. 1. Coomassie blue-stained SDS–PAGE of soluble proteins from chloroplasts. All resolving gels were 12.5% (C = 2.5%) with 5% stacking gels. Lane 1, the gel was run according to the standard Laemmli procedure (2); lane 2, the gel was cast according to the Laemmli procedure but prerun as described by Applied Biosystems User Bulletin No. 25 (4); lane 3, the gel was prerun by the buffer exchange method described above; lane 4, BDH protein standards.

that the buffer exchange procedure described in this paper results in a resolution similar to that obtained when gels were not prerun.

The resolution of the Laemmli procedure for polyacrylamide gels depends on concentration of the sample into a narrow band at the interface of the stacking and running gel as a consequence of the relative rates of migration of the glycine and chloride ions through the stack (3). Prerunning gels results in a loss of the concentration/pH boundary at the base of the stacking gel and replaces the chloride in the stacking gel with glycine so that no glycine/chloride boundary moves through the stacking gel during electrophoresis. While the buffer exchange procedure described above does not completely replace these boundaries, prerunning with stacking gel buffer in the upper reservoir partially restores the pH/concentration boundary in the stacking gel and retains the chloride ion in the stacking gel permitting the formation of a glycine/chloride boundary during the analytical run and the retention of the resolution.

In conclusion, casting the stacking gel with running gel buffer rather than the normal stacking gel buffer and the subsequent electrophoretic introduction of stacking gel buffer during the prerun greatly improves the resolution of prerun gels without involving any additional reagents other than those which would normally be available. Routine use of this simple method has shown it is reliable and can be used to obtain bands for protein sequencing that would otherwise be lost. However, it must be stressed that in addition to obtaining good resolution during the gel separation, the source of acrylamide used for the gels is critical if high initial yields are to be obtained during subsequent gas-phase sequencing of blotted proteins. Experience has shown that with selected acrylamide and a second free radical scavenger (100 μM thioglycollate) present during electrophoresis (4), initial yields of 90% can be expected for standard proteins such as protein A, whereas without prerunning an initial yield of about 60% is more likely. Acrylamides from BDH Chemical Co. and Fluka Chemicals Ltd. (Gillingham, Dorset, UK) have both proved satisfactory for protein sequencing provided that each batch is checked for the initial yield using a standard protein.

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Liquid Scintillation Counting of Tritium-Labeled Neuropeptide in the Subnanomole Range: Quantitative Study of Adsorption to Vials[1]

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Neuropeptides tend to be adsorbed to the walls of containers in which they are kept, and the degree of adsorption depends on factors such as the container material and the concentration of the peptide solution (1,2). Although these problems have been known for some time and most workers in the field take some precautionary steps to minimize the loss of peptides in their experiments, few quantitative data are available, and it is in fact difficult to obtain such data unless the neuropeptides are available in radiolabeled form. Obviously, the loss from defined solutions will affect the actual neuropeptide dosage in physiological or biochemical experiments. The adsorption can also compromise quanti-

[1] Mention of a company name of proprietory product does not constitute an endorsement by the U.S. Department of Agriculture.

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tative determinations of neuropeptides by liquid scintillation counting because the adsorbed peptide will be counted with a different (less efficient) counting geometry than the peptide in solution, resulting in underestimation of the quantity (3). The aim of the present study was to determine the conditions for reproducible liquid scintillation counting of neuropeptide samples applied to or obtained from metabolic experiments. We have employed a biologically active analog (NLPBAN)[3] of *Helicoverpa zea* PBAN (4,5). The results should be applicable to other peptides of similar hydrophobicity and charge.

Chemicals. NLPBAN, a 33-residue C-terminally amidated peptide (molecular weight 3900), was obtained by catalytic tritiation (Dupont NEN Research Products, Boston, MA) or hydrogenation of iodo-NLPBAN ($I_2Tyr^{15,28}$-NLPBAN) to yield tritiated or unlabeled NLPBAN, respectively,[4] and the molecular weight was confirmed by electrospray mass spectrometry.

Liquid scintillation counting. Duplicate samples were prepared by first measuring various volumes of solvent[5] as indicated, into 7-ml Pico glass vials with aluminum-lined caps (Packard, Downers Grove, IL) or 5-ml polypropylene scintillation vials with polyethylene caps (Kimble, Vineland, NJ). In some experiments the solvent contained BSA or unlabeled NLPBAN. Aliquots of [^{3}H]NLPBAN in 15 or 50 μl solvent were then transferred into the previously measured solvent and mixed gently. Finally, Ecoscint A[6] was added (2.5 or 4 ml to polypropylene or glass vials, respectively, to obtain an equal liquid column height), and the samples were shaken vigorously. The samples were counted five times in 6-h intervals (beginning 1 h after mixing with Ecoscint A) in a Packard Tri-Carb Model 460C liquid scintillation system (equipped with a radium-226 source for external standardization). Results are based on dpm to correct for variations in the counting efficiency (46–57%) caused by addition of various volumes of solvent. Cpm–dpm conversion was based on a curve generated with sealed tritium Pico Standards (Packard). The results ("dpm yields") are expressed as percentages of the maximum dpm obtained for a given aliquot of [^{3}H]NLPBAN stock solution (in the absence of significant adsorption to vial walls).

Effects of sample volume and BSA. BSA, other proteins, or peptides have been used as an additive to aqueous neuropeptide solutions or for pretreatment of equipment (1,2,7,8) with the expectation that these substances will saturate potential peptide binding sites on the vial walls and thereby prevent the loss of peptide from the sample solutions. To determine whether BSA addition may also be advantageous for liquid scintillation counting of peptides, samples containing [^{3}H]-NLPBAN in 100, 200, or 500 μl of solvent, with or without BSA (200 μg/ml), were prepared and counted. The highest and most uniform counting rates were obtained with polypropylene vials (Fig. 1B), and there were no significant differences between the various solvent volumes. Samples in glass vials containing 200 or 500 μl solvent also yielded very uniform high counting rates (Fig. 1A), whereas the rates of samples with only 100 μl solvent in glass were lower in the first counting cycle and decreased over time. Samples containing BSA additions (Figs. 1C,1D) showed greater volume-related variations in the counting rates than samples without BSA. The rates of the BSA-containing samples increased slightly with increasing solvent volumes, but even at the highest volume the rates were no better than those of BSA-free samples. The amounts of BSA in the samples ranged from 10 to 90 μg depending on the amount of BSA-containing solvent mixture added (50, 150, or 450 μl), providing for a 7- to 63-fold excess over the NLPBAN (1.42 μg).

Effects of unlabeled NLPBAN. Figure 2 shows the effect of various amounts of unlabeled NLPBAN on the dpm yields of [^{3}H]NLPBAN samples in glass or polypropylene vials. Prior exposure of the vial walls to and presence of unlabeled NLPBAN did not increase the dpm yields in either type of vial. The presence of 500 μl solvent in the samples apparently assured optimum counting rates (i.e., negligible adsorption) with as little as 0.12 μg of peptide. In contrast, samples containing only 15 μl of [^{3}H]NLPBAN solution and Ecoscint A, but no additional solvent, yielded only 86–91% of the total dpm, suggesting a substantial loss of dissolved [^{3}H]NLPBAN due to adsorption.

[3] Abbreviations used: BSA, bovine serum albumin; NLPBAN, bis-norleucine analog ($Nle^{5,14}$) of *Helicoverpa zea* pheromone biosynthesis-activating neuropeptide (Leu-Ser-Asp-Asp-Nle-Pro-Ala-Thr-Pro-Ala-Asp-Gln-Glu-Nle-Tyr-Arg-Gln-Asp-Pro-Glu-Gln-Ile-Asp-Ser-Arg-Thr-Lys-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2); PBAN, pheromone biosynthesis-activating neuropeptide; TFA, trifluoroacetic acid.

[4] [^{3}H]NLPBAN, unlabeled NLPBAN, or mixtures thereof were purified by HPLC on a Delta Pak C_{18}-300 Å (3.9 mm × 15 cm, 5 μm) column (Millipore) or a VYDAC C_4 (1 × 25 cm, 5 μm, 300 Å) column (The Nest Group, Southborough, MA) using acetonitrile/water gradients with 0.1% TFA in each solvent (20–30% or 18–24% acetonitrile for Delta Pak or VYDAC, respectively). Judged by its elution from the VYDAC C_4 column in a slow acetonitrile/H_2O/0.1% TFA gradient with 22–23% acetonitrile, the hydrophobicity of NLPBAN is between that of *Manduca sexta* allatotropin (eluted with 15–16% acetonitrile) (2) and *M. sexta* diuretic hormone (eluted with 28–29% acetonitrile) (6). Quantification of NLPBAN was based on the uv absorption of HPLC effluents (peak area at 214 nm; Waters Model 810 baseline workstation). The concentration of the NLPBAN standard solution used for the calibration of the HPLC system was determined by OPA-Fmoc amino acid analysis.

[5] Water/acetonitrile/TFA (80/20/0.1, by vol; pH ~2) was used as solvent for NLPBAN throughout this study.

[6] A liquid scintillation cocktail (70–85% chiral phenylalkanes, 15–30% nonionic surfactants; National Diagnostics, Manville, NJ) with a high holding capacity for aqueous samples.

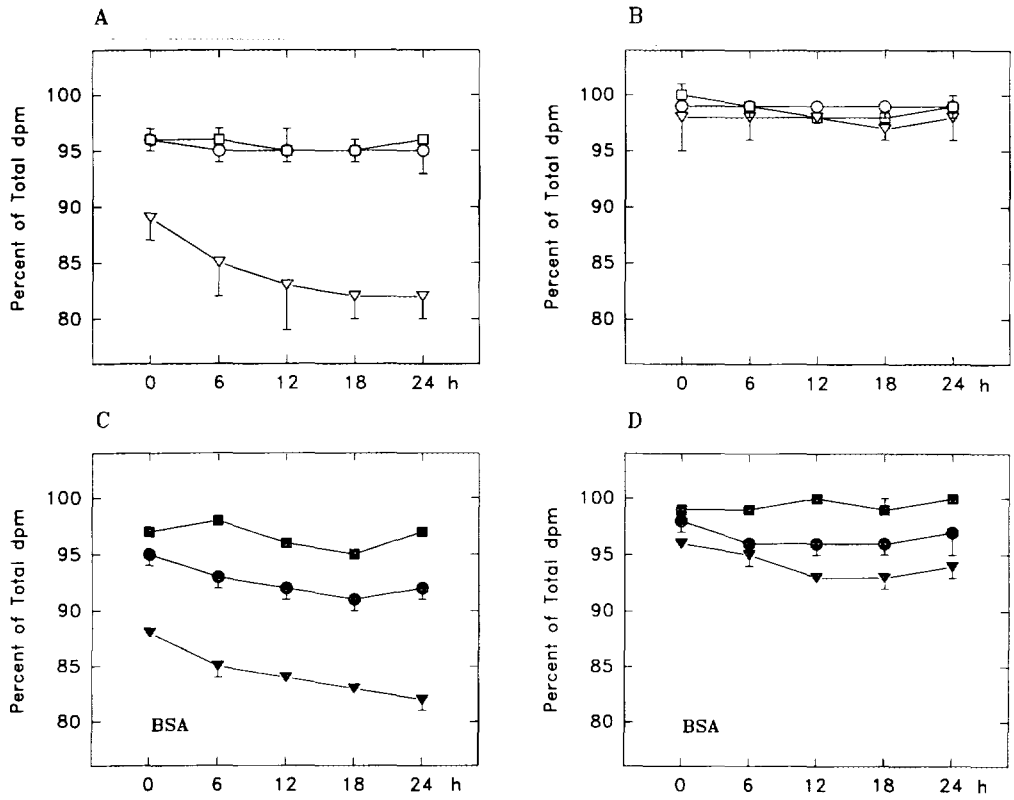

FIG. 1. Dpm yields (means ± SD) of [^{3}H]NLPBAN samples in various volumes of solvent (water/acetonitrile/TFA, 80/20/0.1), with (C, D) or without (A, B) BSA, in glass (A, C) or polypropylene (B, D) vials. [^{3}H]NLPBAN (10,300 dpm; 1.42 μg = 0.37 nmol) in 50 μl solvent was measured into 50, 150, or 450 μl of solvent (±200 μg BSA/ml) to give 100 (▽, ▼), 200 (○, ●), and 500 μl (□, ■) total solvent volume.

To obtain direct proof for the adsorption, selected samples were transferred to fresh vials and the radioactivity in the transferred samples and in the emptied vials was determined (Table 1). Samples without added solvent lost another 7–8% of the total dpm (8–9% of the transferred dpm) in the fresh vials, and the emptied original vials contained 11–12% of the total dpm (equal to the original dpm shortfall). These data indicate that

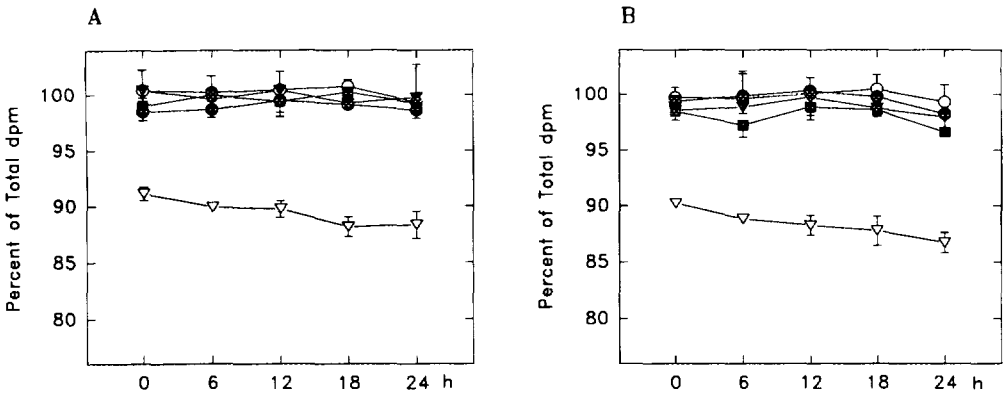

FIG. 2. Dpm yields (means ± SD) of [^{3}H]NLPBAN samples in glass (A) or polypropylene (B) vials containing various amounts of unlabeled NLPBAN. Different amounts of NLPBAN (0.75, 1.5, or 3.0 μg) were measured into solvent to give a final volume of 500 μl. Vial walls were wetted carefully to the final filling levels (4.5 or 3.0 ml, for glass or polypropylene vials, respectively). Control vials contained only 500 μl solvent. Next, [^{3}H]NLPBAN (11,300 dpm; 0.12 μg = 29.4 pmol; in 15 μl solvent) was dispensed into these solutions, followed by addition of Ecoscint A, mixing, and counting. For one group of samples solvent was omitted and [^{3}H]NLPBAN was dispensed directly into Ecoscint A. (○) Solvent + [^{3}H]NLPBAN (0.12 μg); (▼) solvent + 0.75 μg NLPBAN + [^{3}H]NLPBAN; (●) solvent + 1.5 μg NLPBAN + [^{3}H]NLPBAN; (■) solvent + 3.0 μg NLPBAN + [^{3}H]NLPBAN; (▽) no solvent, no NLPBAN, 0.12 μg [^{3}H]NLPBAN only.

TABLE 1

Dpm Yields and Adsorption of [^{3}H]NLPBAN on Walls of Glass or Polypropylene Vials[a]

Sequence of sample preparation	NLPBAN (μg)	Initial vial dpm[b] (% of total)	After transfer dpm[c] (% of total)	Residual dpm[d] (% of total)
		Glass		
Ecoscint A, [^{3}H]NLPBAN	0.12	88.2 ± 0.8	80.3 ± 0.4	10.8 ± 0.0
Solvent, [^{3}H]NLPBAN, Ecoscint A	0.12	100.0 ± 0.9	98.4 ± 0.9	0.5 ± 0.1
Solvent, NLPBAN, [^{3}H]NLPBAN, Ecoscint A	3.12	99.7 ± 0.7	97.9 ± 0.4	0.4 ± 0.1
		Polypropylene		
Ecoscint A, [^{3}H]NLPBAN	0.12	87.2 ± 1.0	80.3 ± 1.7	12.1 ± 0.5
Solvent, [^{3}H]NLPBAN, Ecoscint A	0.12	99.9 ± 1.3	97.6 ± 0.2	0.9 ± 0.1
Solvent, NLPBAN, [^{3}H]NLPBAN, Ecoscint A	3.12	97.6 ± 1.2	97.3 ± 1.2	0.9 ± 0.1

[a] All samples contained [^{3}H]NLPBAN (11,300 dpm; 0.12 μg; in 15 μl solvent), unlabeled NLPBAN (3.0 μg), and/or 500 μl solvent where indicated. After completion of the first five counting cycles, the samples were transferred to new vials of the same material and recounted. The emptied vials were rinsed with 500 μl solvent, followed by Ecoscint A addition and counting ("residual").

[b] Means ± SD of last two counting cycles before sample transfer.

[c] Means ± SD of first two counting cycles after sample transfer.

[d] Means ± SD of first two counting cycles of emptied original vials.

11–12% of the [^{3}H]NLPBAN had been adsorbed by the walls of the original vials and a similar proportion of the peptide was adsorbed again by the walls of the fresh vials after sample transfer. Furthermore, the fraction of the peptide bound to the walls was not just counted at a diminished efficiency, but was not counted at all. In other words, the adsorption of the [^{3}H]NLPBAN molecules to the walls completely prevented the β-energy transfer to the dissolved fluor molecules.

Samples containing 500 μl of solvent, on the other hand, only showed negligible dpm loss as a result of sample transfer, and the emptied original vials also showed only negligible radioactivity, regardless of the presence of added unlabeled NLPBAN or lack thereof. Thus, the solvent addition did indeed prevent or minimize NLPBAN adsorption on the vial walls.

Effects of partial or complete solvent evaporation. Another set of tests was conducted to determine suitable conditions for the preparation of HPLC effluent fractions for liquid scintillation counting. Figure 3 shows the effects of partial (to 0.5 ml) or complete solvent evaporation on the counting rates. All samples yielded counts in the 95–100% bracket. Again the addition of BSA, now present in a higher concentration than in the previous experiments (i.e., 600 μg/sample), did not improve the dpm yields. The partial evaporation in glass was deemed to be the most efficient and time-saving method because of the better heat transfer and more

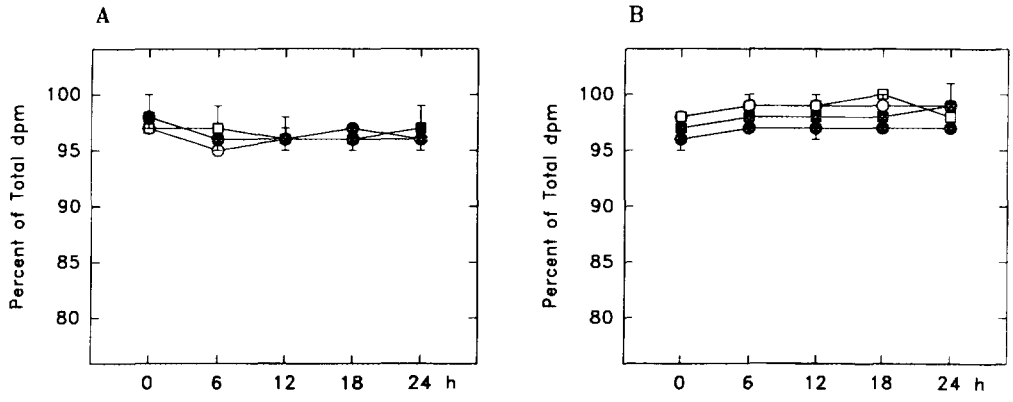

FIG. 3. Dpm yields (means ± SD) of [^{3}H]NLPBAN samples in glass (A) or polypropylene (B) vials after partial or complete evaporation of solvent. Samples of [^{3}H]NLPBAN (10,300 dpm; 1.42 μg) in 3 ml solvent with (●, ■) or without (○, □) 200 μg BSA/ml were either reduced to 0.5 ml (●, ○) or dried completely (■, □) in a Speedvac apparatus (Savant Instruments, Farmingdale, NJ). Completely dried samples were redissolved in 0.5 ml solvent. After addition of Ecoscint A all samples were counted in the same vials in which they had been concentrated or dried.

rapid evaporation compared to polypropylene, and because the residual solvent (water/TFA) eliminated the necessity of renewed solvent addition and redissolving of the peptide.

In conclusion, it has been shown that small amounts (29–370 pmol) of a tritium-labeled neuropeptide can be counted with 95–100% yield in either glass or polypropylene vials provided that ≥200 μl of water/acetonitrile/TFA (80/20/0.1) is mixed with the peptide sample prior to the addition of Ecoscint A and counting. Under these conditions addition of BSA or unlabeled NLPBAN did not improve the counting rates. Partial or complete evaporation of samples in 3 ml of the solvent mixture did not cause significant reduction of the dpm yield. There was no indication of the irreversible loss of peptide as a result of concentrating or drying of solutions reported by other workers (1,2). This difference in the recovery may be related to the differences in the balance between hydrophobic and charged groups among peptides, to the presence of high concentrations of solvents and surfactants (components of Ecoscint A), and probably most importantly to the use of an acidic solvent mixture in our samples.

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A Rapid Protein Determination by Modification of the Lowry Procedure[1]

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The method for protein measurement described by Lowry *et al.* (1) is one of the most commonly used procedures in the laboratory. One of the main disadvantages of this procedure is the length of time involved in completing the assay. The present study demonstrates that incubating the samples at 37°C accelerates the color development, allowing the assay to be performed in a significantly briefer period of time (2).

Materials and methods. Bovine serum albumin (type V), human serum albumin, ovalbumin, trypsin, Folin and Ciocalteau's phenol reagent (2.0 N), were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade. The protein was dissolved in distilled water (concentration 1 mg/ml) and the appropriate concentrations of the protein solution were pipetted into test tubes (final volume 300 μl). To each of these test tubes, 1 ml of alkaline copper sulfate solution (containing 185 mM Na_2CO_3, 98.1 mM, NaOH, 0.39 mM $CuSO_4 \cdot 5H_2O$, and 0.7 mM $KNaC_4H_4O_6 \cdot 4H_2O$) was added, mixed thoroughly, and incubated at the specified temperatures for various periods of time. After incubation, 0.1 ml of Folin and Ciocalteau's reagent (diluted 1:1 v/v with H_2O) was added and mixed immediately. After further incubation, the color developed was read at 750 nm for the protein concentrations of 25 μg/ml and at 500 nm for higher protein concentrations, utilizing a Beckman Du-65 spectrophotometer (Fullerton, CA).

Results and discussion. In the initial experiments the incubation period after the addition of alkaline copper sulfate was kept constant at 3 min while the time intervals following the addition of Folin and Ciocalteau's reagent were varied. In order to confirm

[1] The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Naval Service at large.

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